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Pathogen-Induced Proteins with Inhibitory Activity toward *Phytophthora infestans*

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A bioassay using *Phytophthora infestans* was developed to determine whether inhibitory proteins are induced in pathogen-inoculated plants. Using this bioassay, AP24, a 24-kilodalton protein causing lysis of sporangia and growth inhibition of *P. infestans*, was purified from tobacco plants inoculated with tobacco mosaic virus. Analysis of the N-terminal amino acid sequence identified AP24 as the thaumatin-like protein osmotin II. The sequence was also similar to NP24, the salt-induced protein from tomato. Subsequently, we purified a protein from tomato plants inoculated with *P. infestans* that had inhibitory activities identical to those of the tobacco AP24. The N-terminal amino acid sequence of this protein was also similar to those of osmotin and NP24. In general, both the tobacco and tomato AP24 caused lysis of sporangia at concentrations greater than 40 nanomolar and severely inhibited hyphal growth at concentrations greater than 400 nanomolar. Because both proteins were induced by pathogen inoculation, we discussed the possible involvement of these proteins as a plant defense mechanism.

INTRODUCTION

In many plant species, resistance against infections by pathogenic bacteria, viruses, and fungi can be induced by a variety of biotic and abiotic elicitors (reviewed in Ouchi, 1983; Sequeira, 1983). Examples have been described for, among other species, cucumber, tomato, tobacco, and potato (McIntyre and Dodds, 1979; Jenns and Kuć, 1980; Heller and Gessler, 1986; Doke et al., 1987). The mechanisms responsible for this induction of resistance are not known; however, much research has focused on the proteins that accumulate specifically in the induced plants (Van Loon, 1985; Parent and Asselin, 1987; Carr and Klessig, 1989; Bol et al., 1990). One group of induced proteins, known as pathogenesis-related (PR) proteins, is secreted to the intercellular spaces of leaves, is relatively protease resistant, and is soluble at low pH (Van Loon, 1985). A second group of proteins is related serologically to the PR proteins but accumulates intracellularly in the induced plants (Carr and Klessig, 1989; Bol et al., 1990).

The correlation of the induced resistance response with the accumulation of the two groups of proteins suggests that these induced proteins are part of a plant defense mechanism. This hypothesis was supported by the discovery of several isoforms of chitinase and β-1,3-glucanase among the proteins (Kauffmann et al., 1987; Legrand et al., 1987). Substrates for these enzymes are chitin and β-1,3-glucan, respectively, the major structural components of the cell walls of many fungi. The oligomers of chitin and glucan released by the enzymes are effective elicitors of other resistance responses in plants such as hypersensitivity and the biosynthesis of lignin and phytoalexins (Dean and Kuć, 1987). The observed lysis and growth inhibition of several plant pathogenic fungi in vitro caused by chitinase and β-1,3-glucanase (Schlumbaum et al., 1986; Mauch et al., 1988) suggest that the induced proteins are also capable of acting directly on the invading pathogen in vivo. Despite all of these observations, there is no direct evidence in vivo indicating the involvement of the induced chitinases and β-1,3-glucanases in resistance against fungi.

Cell walls of most fungi in the taxonomic class Oomycetes have a β-1,3-glucan component but contain no chitin. In vitro, the Oomycetes *Phytophthora cactorum*, *Pythium ultimum*, and *Pythium aphanidermatum* were shown to be insensitive to a mixture of chitinase and β-1,3-glucanase (Mauch et al., 1988). Considering that resistance against Oomycetes is inducible in several plant species (McIntyre and Dodds, 1979; Cohen and Kuć, 1981; Heller and Gessler, 1986; Doke et al., 1987), we hypothesized that factors other than chitinase and β-1,3-glucanase are involved in induced resistance against this class of fungal pathogens. In the present study, the experimental aim was the identification of such factors. To this end, a bioassay was developed using *P. infestans*, an agronomically important pathogen, which causes late blight disease of potato and tomato. Using this bioassay, we detected an activity in extracts of pathogen-induced tobacco and tomato plants

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causing lysis of sporangia and inhibition of hyphal growth. Here we present the purification and characterization of an induced protein (AP24) having these inhibitory activities.

RESULTS

Bioassay

To detect proteins inhibitory to the growth of *P. infestans*, a bioassay was developed using microtiter dishes. In this assay, protein solutions were dialyzed against the proper buffer and added to a suspension containing sporangia of *P. infestans*. After incubation for 4 days to 5 days, the development of mycelium was taken as a measure of growth. This method facilitated the testing of the numerous fractions obtained during the purification process. To set up the test system, various parameters needed to be optimized because the germination and growth of *P. infestans* were inhibited by most buffers at relatively low molarity. As shown in Table 1, phosphate buffers at pH 7 were more inhibitory than at pH 6. Concentrations of NaOAc (pH 5.2) as low as 1 mM were extremely inhibitory. NaCl at concentrations below 25 mM had no effect on fungal growth and was only slightly inhibitory up to 100 mM. β-Mercaptoethanol was inhibitory at concentrations of 0.5 mM and higher. None of the buffer components tested caused lysis of germinating sporangia. Initially, we determined the optimum buffer conditions for the bioassay as 5 mM $KHPO_4$, pH 6. However, during the process of purification, proteins and the antifungal activity were lost, probably because of the low ionic strength of this buffer. Subsequently, the assay buffer was adapted to 15 mM $KHPO_4$, pH 6, 20 mM NaCl, which was the maximum buffer concentration and ionic strength having no effect on fungal growth.

In the microtiter dish bioassay, the populations of sporangia were not synchronized in germination or growth. There was variability between experiments in germination levels and growth rates; however, no difference in these characteristics was observed between the sporangial populations in the different wells within experiments.

Table 1. Effect of Various Buffer Components on the Growth of *P. infestans* in Vitro[a]

Buffer	Minimum Inhibitory Concentration	100% Inhibition of Germination
NaOAc pH 5.2	1 mM	10 mM
$NaHPO_4$		
pH 6.0	20 mM	>50 mM
pH 7.0	10 mM	25 mM
$KHPO_4$		
pH 6.0	20 mM	>50 mM
pH 7.0	10 mM	25 mM
NaCl	25 mM	>100 mM
β-Mercaptoethanol	0.5 mM	5 mM

[a] Growth inhibition was determined by comparison with a water control after 5 days' incubation.

Identification and Purification of Inhibitory Activity

Leaves of Samsun NN tobacco plants were inoculated with tobacco mosaic virus (TMV). This treatment induces a resistance against the fungus *P. nicotianae* (McIntyre and Dodds, 1979; C.P. Woloshuk and M. Sela-Buurlage, unpublished results). After incubation for 7 days to allow necrotic lesion formation, total leaf proteins were extracted and passed over a Sephadex G-25 column. Using the bioassay, an inhibitory activity toward *P. infestans* was first detected in this G-25 fraction. The activity was observed as the lysis of sporangia after an overnight incubation (18 hr to 20 hr) and reduced growth in the microtiter dish after 4 days' to 5 days' incubation. This activity was inactivated completely by heat treatment (100°C for 10 min). An extract was also obtained from noninduced tobacco leaves. The concentrated G-25 fraction from this extract contained no lysis or growth inhibiting activity.

When the G-25 fraction was passed over an S-Sepharose column, no inhibitory activity was detected in the unbound protein fraction. The adsorbed proteins were eluted from the column and every third fraction was tested for chitinase, β-1,3-glucanase, and antifungal activity. This analysis, shown in Figure 1, indicated that the inhibitory activity (fractions 56 to 62) eluted between the peak fractions of chitinase (fraction 41) and β-1,3-glucanase (fraction 74). Mixtures of chitinase and β-1,3-glucanase are known to have antifungal activity (Mauch et al., 1988); therefore, a comparison was made of the peak inhibitory fraction with a mixture of the peak chitinase and β-1,3-glucanase fractions. As shown in Figure 2, virtually no inhibitory activity was detected in this mixture even though the β-1,3-glucanase activity was 4.6 times higher and the chitinase activity was 1.3 times higher than in the fraction containing the inhibitory activity. These results are in line with observations that Oomycetes appear insensitive to a mixture of chitinase and β-1,3-glucanase (Mauch et al., 1988). In addition, these results confirm the idea that factors with antifungal activity other than chitinase and β-1,3-glucanase are induced in tobacco by infection with TMV.

As shown in Figure 3, the inhibitory activity was abolished completely after incubation with proteinase K, indicating that the inhibitory activity was proteinaceous. SDS-PAGE analysis indicated that all proteins in the fraction were degraded by the proteinase (data not shown).

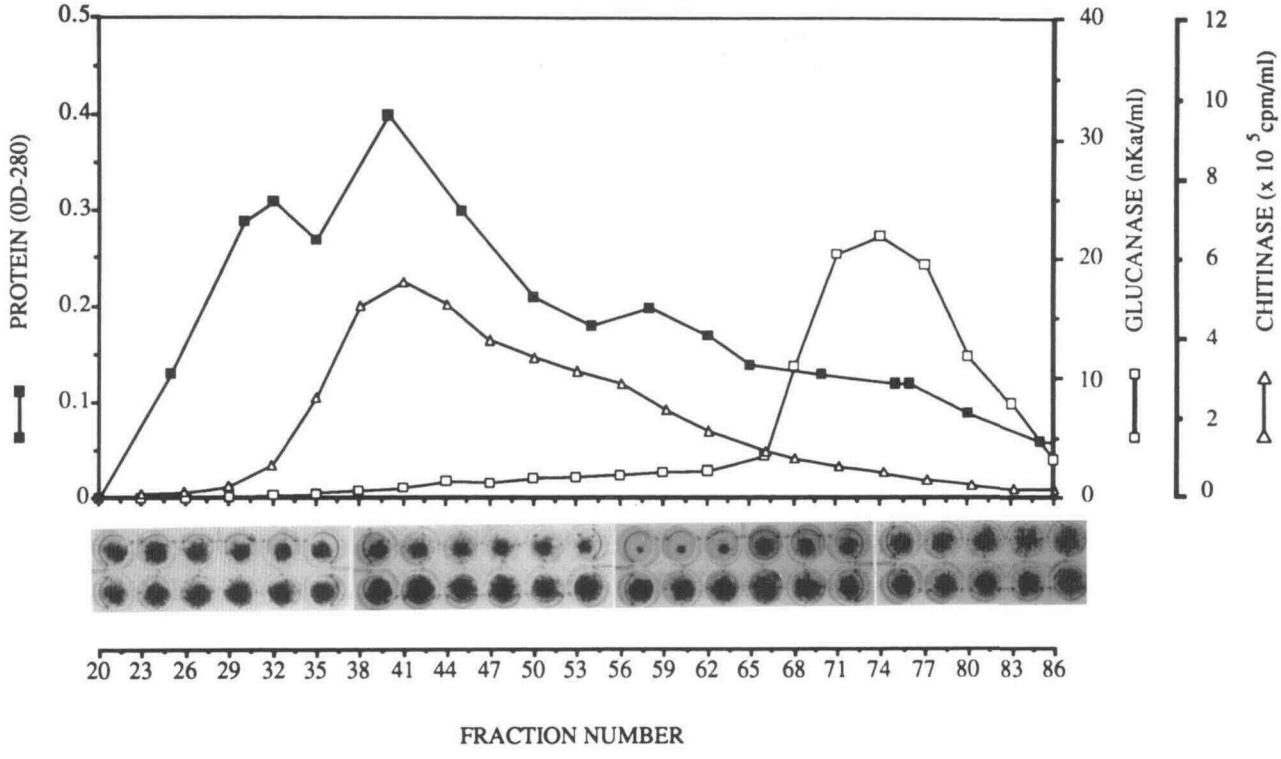

Figure 1. S-Sepharose Chromatography of Tobacco Proteins Obtained from G-25 Filtration.

Protein was measured as optical density at 280 nm. Chitinase was determined by measuring the amount of soluble radioactivity released from ^{3}H-labeled chitin after 30-min incubation. Glucanase was determined using laminarin as a substrate. For the bioassay, 0.1 mL of each fraction was added to the bioassay wells (top row). As controls, corresponding fractions were heat treated for 10 min at 100°C (bottom row). Mycelia were stained with lactophenol cotton blue and photographed after 5 days' incubation.

As for the controls, treatment of the active fraction with denatured proteinase K under the same conditions had no effect on the inhibitory activity, and the addition of proteinase K alone to the bioassay had no effect on the growth of *P. infestans*.

For further purification, the S-Sepharose fractions containing inhibitory activity were pooled and loaded onto a hydrophobic interaction column (phenyl-superose). The inhibitory activity eluted from the column at the end of the elution gradient, indicating its very hydrophobic character. This area is illustrated in Figure 4A. By rechromatographing the active fraction over this same column, an essentially pure protein was obtained having both lysis and growth-inhibiting activities. The protein was called AP24. From the SDS-PAGE analysis shown in Figure 5, the molecular mass of AP24 was calculated as 24 kD. The protein had no β-1,3-glucanase or chitinase activities. The approximate yield of AP24 from 400 g of leaves was 500 μg after the first run on the hydrophobic interaction column, with a loss of half this amount of protein (250 μg) after the second run.

Identification of Tobacco AP24 as Osmotin

To identify AP24 further, the N-terminal amino acid sequence of the protein was determined. The identity of 40 amino acids obtained from this analysis is shown in Figure 6. The elucidated sequence showed complete identity to the N terminus of osmotin II (Figure 6), a tobacco protein that accumulates in response to high NaCl stress (King et al., 1986; Singh et al., 1987a, 1987b, 1989). There are two known isoforms of osmotin (Singh et al., 1987a). The N-terminal sequence of AP24 differed from that of osmotin I at amino acid position 38 only (Figure 6). In addition, only one difference was found with the N-terminal primary sequence of NP24 (position 27, Figure 6), a tomato protein also associated with high NaCl stress (King et al., 1988). A lower degree of homology was found with the bifunctional α-amylase/trypsin inhibitor, MAI, of maize (Richardson et al., 1987), with thaumatin, a sweet-tasting protein from *Thaumatococcus daniellii* (Edens et al., 1982), and with the (extracellular) PR proteins PR-5 of tobacco (Cornelissen et al., 1986; Pierpoint et al., 1987), protein C of

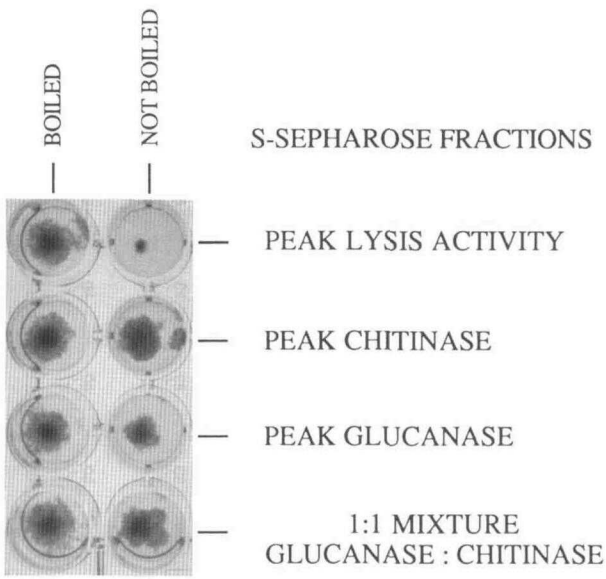

Figure 2. Effect of Chitinase and Glucanase Levels on the Growth of *P. infestans*.

For the bioassay, 0.1 mL of the peak fractions from the S-Sepharose column (see Figure 1) was added to the bioassay wells. The fractions tested were as follows: peak lysis activity, fraction 56; peak chitinase, fraction 41; and peak glucanase, fraction 74. For the 1:1 mixture, 0.05 mL of fractions 41 and 74 were added. As controls (Boiled), corresponding fractions were heat treated for 10 min at 100°C.

potato (Pierpoint et al., 1990), and protein Hv-1 of barley (Bryngelsson and Gréen, 1989).

Antifungal Activity of Tomato AP24

The identification of AP24 as osmotin, together with the observation of the induction of resistance against *P. infestans* in tomato, prompted us to extend our studies to tomato. Resistance to *P. infestans* was induced in tomato according to Heller and Gessler (1986). After induction, a total leaf extract was analyzed for antifungal activity in the bioassay using *P. infestans*. An inhibitory activity similar to that caused by the tobacco AP24 was detected in this extract. Subsequently, the same purification scheme was used as described for tobacco and the activity chromatographed similar to the tobacco AP24. One difference was that the inhibitory activity from tomato eluted from the hydrophobic interaction column earlier than the tobacco proteins (Figure 4B). A protein of 24 kD was subsequently purified to homogeneity (Figure 5) and N-terminal amino acid sequencing indicated that the protein was similar to NP24 (King et al., 1988) with amino acid differences at positions 3 and 27 (Figure 6). This result indicated that tomato NP24, like tobacco osmotin, inhibits the growth of *P. infestans* in vitro.

Immunological Analysis

Because of the sequence similarity of the tobacco AP24 and the tomato AP24 with the tobacco PR protein PR-5, the serological relationship was analyzed using an available antiserum produced against PR-5. Immunological analysis indicated that the PR-5 antiserum recognizes both AP24 proteins. Using this antiserum, an immunoblot of total protein extracts from induced and noninduced tobacco and tomato plants was tested. As shown in Figure 7, there was a dramatic increase in the level of AP24-like proteins after induction. Because tobacco PR-5 migrated on gel with AP24, no conclusion can be made as to the level of induction of either protein. We believe AP24 accounts at least in part for the observed band on the immunoblot because the antifungal activity identified as AP24 was present in the G-25 fraction of the TMV-inoculated tobacco leaves, and not in the G-25 fraction from healthy plants. We have also run the extracts from control plants through the same purification scheme as the TMV-induced plants

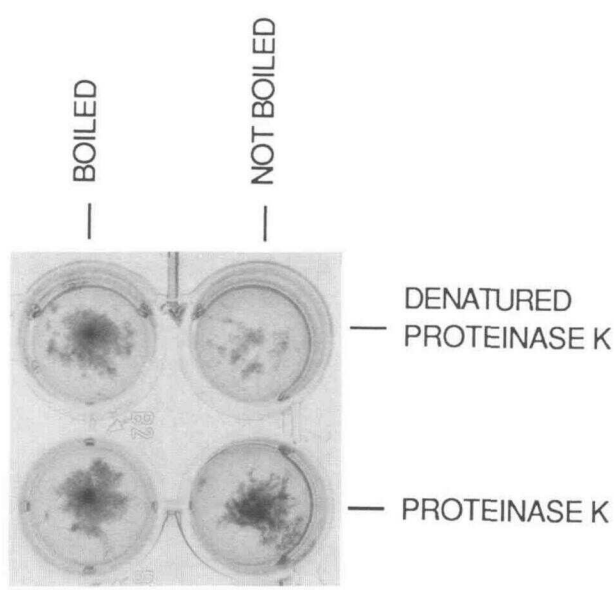

Figure 3. Effect of Proteinase K Treatment on the Inhibitory Activity Obtained from the S-Sepharose Column (Figure 1).

The fractions with inhibitory activity were treated for 15 hr with 1.5 mg/mL proteinase K or heat-denatured proteinase K. Before adding to the wells of the bioassay, 0.1 mL of each treatment was boiled (10 min) or not boiled. The photograph was taken after 5 days' incubation.

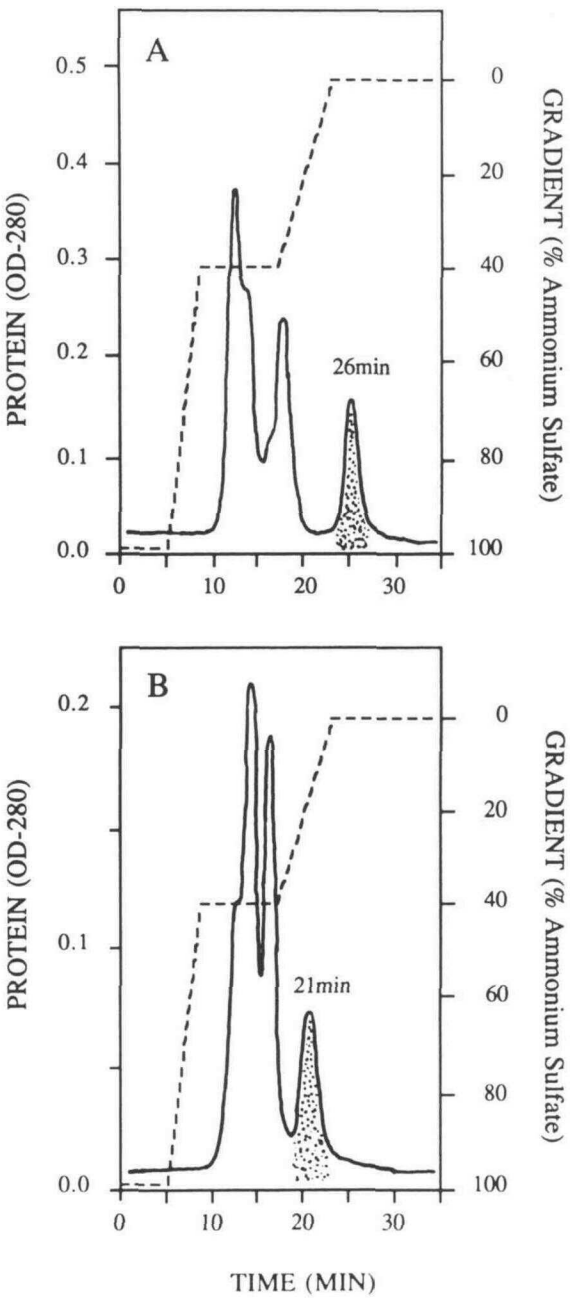

Figure 4. Purification of AP24 by Phenyl-Superose Chromatography.

(A) Tobacco.
(B) Tomato.
Those fractions from the S-Sepharose column (Figure 1) with inhibitory activity toward *P. infestans* were applied to the phenyl-superose column. Protein was measured as optical density at 280 nm. Each fraction was tested in the bioassay as in Figure 1. The shaded area designates the fractions containing inhibitory activity.

and detected no or in some experiments only low levels of AP24. Furthermore, we have purified PR-5 from the TMV-inoculated leaves and found that 50 μg/mL PR-5 had no antifungal activity (results not shown). This reasoning is supported also by the recent finding of induction of osmotin mRNA synthesis in tobacco upon inoculation with TMV (Neale et al., 1990; H.J.M. Linthorst and J.F. Bol, unpublished results).

Biological Activity of AP24

In pure form, AP24 frequently precipitated during dialysis to the buffer conditions needed in the bioassay. Despite losses as high as 50%, sufficient amounts of tobacco AP24 and tomato AP24 were attainable to determine the effects on *P. infestans*. Inhibition of the fungus was a combination of two effects: lysis of the sporangia and inhibition of hyphal growth.

At 1 μg/mL, tobacco AP24 caused significant lysis of the sporangial population. The percentage of lysed sporangia increased with the increasing AP24 concentration; 59% and 72% of the sporangia were lysed by AP24 concentrations at 5 μg/mL and 20 μg/mL, respectively. Lysis in the control was 10%. Lysis was always observed at the apex of the sporangia where the germ tube normally emerged. This lysis is illustrated in Figure 8A. Lysis of

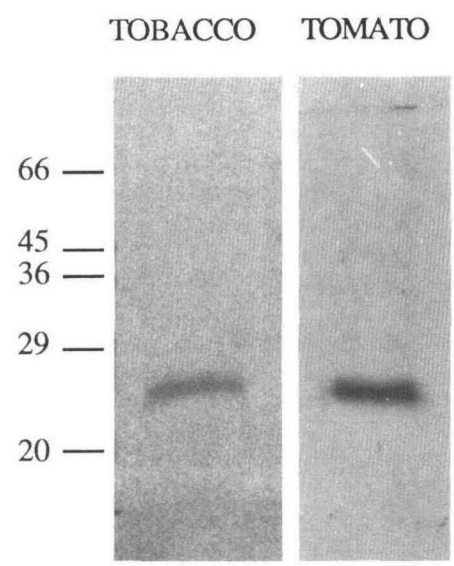

Figure 5. SDS-PAGE of Tobacco and Tomato AP24 Obtained from the Phenyl-Superose Column.

The migration of the molecular mass markers is indicated on the left in kilodaltons. The gel was stained with Coomasie Brilliant Blue.

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                                                    *
Tobacco AP24    ATIEVRNNCPYTVWAASTPIGGGRRLDRGQTWVINAPRGT
Osmotin I       ATIEVRNNCPYTVWAASTPIGGGRRLDRGQTWVINAPPGT
Osmotin II      ATIEVRNNCPYTVWAASTPIGGGRRLDRGQTWVINAPRGT
                  *                         *
Tomato AP24     ATFEVRNNCPYTVWAASTPIGGGRRLD
Tomato NP24     ATIEVRNNCPYTVWAASTPIGGGRRLNRGQTWVINAPRGT
                1         10        20        30        40
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Figure 6. Comparison of the N-Terminal Amino Acid Sequences of the Tobacco AP24 with Osmotin I and II, and the Tomato AP24 with Tomato NP24.

An asterisk (*) above the amino acid designates a difference among the compared sequences.

hyphal tips occurred only at the higher concentrations (40 μg/mL). Lysis was maximal 18 hr to 24 hr after mixing the sporangia with AP24, corresponding to the time of sporangial germination. When AP24 was added after sporangia were first incubated for this period of time (18 hr), lysis only occurred in those sporangia that had not germinated before the addition of AP24.

In the bioassay, a percentage of the sporangia treated with AP24 always germinated without lysis. The growth of these was affected, however, by the AP24 concentration. At 1 μg/mL tobacco AP24, the germinated sporangia appeared to have the same growth rate as the control. Also, after 5 days' incubation, there was no detectable difference in colony size from the control. A concentration of 5 μg/mL had an inhibitory effect on the growth rate; however, quantitation of this growth inhibition was difficult because of hyphal branching and entanglement. After 5 days' incubation, the colony size was smaller than the controls. At concentrations of 10 μg/mL and higher, hyphal growth was severely inhibited, and the resulting inhibition after 4 days' incubation is shown in Figure 9A.

In contrast, pure tomato AP24 appeared to be less active than the tobacco AP24. Lysis of sporangia was observed at the same concentrations as the tobacco AP24 (Figure 8B), but the percentage of lysis was much less; only 22% at 20 μg/mL. Inhibition of hyphal growth was also less than the tobacco AP24 (Figure 9B).

DISCUSSION

Characterization of the Inhibitory Proteins

In the present study, a bioassay was developed for detecting proteins inhibitory to the germination and growth of *P. infestans*. This assay was used subsequently to follow the purification of an inhibitory activity found in TMV-induced tobacco leaves. This activity caused lysis of germinating sporangia and inhibition of hyphal growth. Purification revealed that a 24-kD protein (AP24) was responsible for this activity. N-terminal amino acid sequence analysis indicated that AP24 belongs to a group of proteins that are similar to thaumatin, a sweet-tasting protein from *T. daniellii* (Edens et al., 1982). The protein was identified as an osmotin and, based on the amino acid at position 38 (arginine), it is possibly osmotin II (Singh et al., 1987a, 1989). The hydrophobic nature and a pI value greater than 9.5 (C.P. Woloshuk and E.J.S. Meulenhoff, unpublished results) further support this identification. The estimated molecular mass was different from that reported for osmotin (26 kD, Singh et al., 1987a; King et al., 1988), suggesting that AP24 is a different isoform of osmotin. This is unlikely, however, because the published nucleotide sequence for an osmotin cDNA codes for a 24-kD mature protein, and within the sequence there are no obvious glycosylation sites (Singh et al., 1989).

Based on the purification scheme of the tobacco AP24, a protein having similar chromatographic properties and inhibitory activity was purified from tomato leaves inoculated with *P. infestans*. The tomato AP24 was also hydrophobic but less than the tobacco AP24. N-terminal amino acid analysis showed that this protein was similar to the tomato protein NP24, a previously described protein that has 91% identity in amino acid sequence to osmotin (King et al., 1988). The sequence of the tomato AP24 was

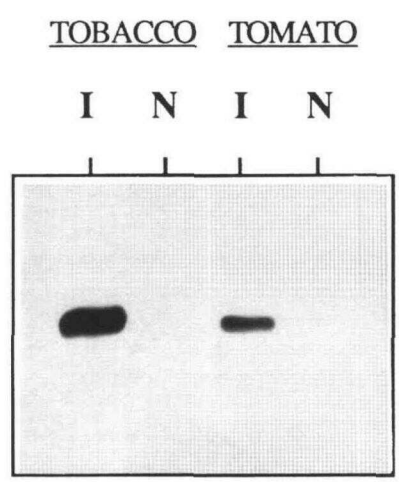

Figure 7. Immunoblot Showing the Induction of AP24-like Proteins in Tobacco and Tomato.

Total proteins were extracted from tobacco and tomato, induced (I) with TMV and *P. infestans*, respectively. The noninduced (N) plants were treated with water. The blot was incubated with tobacco PR-5 antiserum, and the adsorbed antiserum was detected by horseradish peroxidase linked to goat anti-rabbit serum.

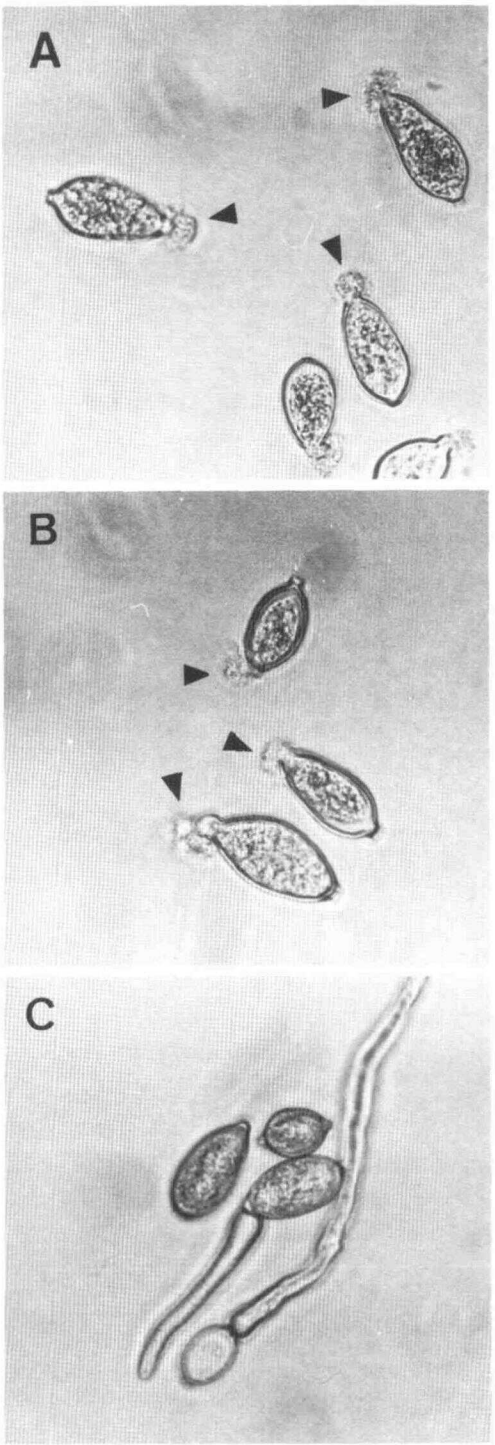

Figure 8. Lysis of Sporangia of *P. infestans* Caused by AP24.

(A) Effect of 10 μg/mL tobacco AP24.
(B) Effect of 10 μg/mL tomato AP24.
(C) Effect of 10 μg/mL boiled tobacco AP24.
The photographs were taken 24 hr after treatment. Arrows indicate the area of lysis.

different from NP24 at amino acid positions 3 and 27, suggesting that there are two isoforms of NP24 in tomato, analogous to osmotin I and II in tobacco. We have identified a second tomato AP24 isomer by immunoblotting that appears less hydrophobic.

Induction of Osmotin-like Proteins in Plants

Osmotin and NP24 were purified previously from tobacco and tomato cell cultures adapted for growth in media

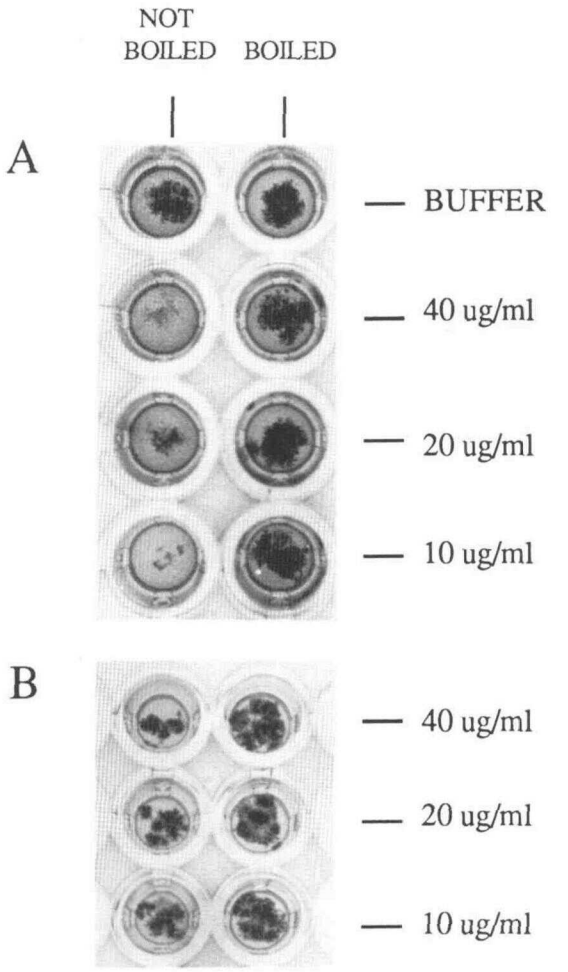

Figure 9. Effect of Various Concentrations of AP24 on the Growth of *P. infestans*.

(A) Effect of tobacco AP24.
(B) Effect of tomato AP24.
Each dilution was added to the bioassay as a boiled (10 min) or not boiled treatment. Mycelia were stained with lactophenol cotton blue and photographed after 5 days' incubation.

containing high concentrations of NaCl (King et al., 1986, 1988). Studies on tobacco cell cultures showed that osmotin accumulated to as much as 12% of the total cellular protein and that the addition of abscisic acid enhanced both the accumulation of osmotin and the adaptation process. In whole plants, the data are somewhat conflicting as to which tissues are most affected by the NaCl or abscisic acid treatment; however, both the root and stem tissues accumulate osmotin in response to such treatment (King et al., 1986; Singh et al., 1987b, 1989). In the present study, an immunoblot indicated that AP24 accumulated in pathogen-induced tobacco and tomato leaves. These data suggest that osmotin is not specific for osmotic stress but is associated with a general stress response in plants. This conclusion is supported further by recent publications showing the biosynthesis of not only osmotin but also other stress-related proteins in tobacco mesophyll cells after protoplast formation (Grosset et al., 1990a, 1990b) and in TMV-infected tobacco (Neale et al., 1990). Considering these observations, we question the appropriateness of the name osmotin.

A group of serologically related proteins having amino acid sequences similar to osmotin also have been shown to accumulate in response to stress. However, these proteins differ from osmotin with respect to their pI values and their extracellular localization. Included in this group are the PR proteins in tobacco (PR-5, Cornelissen et al., 1986; Pierpoint et al., 1987), potato (protein C, Pierpoint et al., 1990), and barley (Hv-1, Bryngelsson and Gréen, 1989). The PR-5 protein of tobacco has 62% amino acid identity with osmotin; however, purified PR-5 was not inhibitory in the *P. infestans* bioassay. Whether the other PR proteins are also not active remains to be determined.

Mechanism of Action

How AP24 inhibits the growth of *P. infestans* is not apparent from the data presented. It is clear that the overall inhibition observed is a combination of the lysis effect and growth inhibition. Because osmotin (AP24) contains 16 cysteine residues that were reduced by the β-mercaptoethanol in the extraction buffer, one might hypothesize that the inhibitory activity was nonspecific, resulting from a high molar concentration of reduced cysteines. We found that PR-5, which also contains 16 cysteines and was purified from the same extracts as AP24, did not cause lysis or inhibit the growth of *P. infestans*. Furthermore, β-mercaptoethanol did not inhibit the growth at concentrations less than 500 μM and did not cause lysis of the sporangia even at the highest tested concentration of 10 mM. These data indicate that if the cysteines of AP24 remained reduced after the initial extraction, they are not responsible for the inhibition of the fungus.

The observed lysis at the sporangial apex suggests that the protein has a cell wall hydrolysis activity. Similar effects have been observed with other fungi when treated with chitinase or β-1,3-glucanase (Mauch et al., 1988). The apex of the sporangia appears to be a weak point in the sporangia of *P. infestans*. As many as 10% of the sporangial population were lysed without the addition of AP24, and this lysis always occurred at the apex area. Another hypothesis is that AP24 interacts with the plasma membrane because of its hydrophobic nature. Such an interaction could disrupt the membrane of *P. infestans*, causing lysis and inhibition of hyphal growth. This hypothesis is supported by the fact that the less hydrophobic tomato AP24 was less active against *P. infestans*.

Role of AP24 in Defense against Fungi

AP24 was induced in tomato by infection with *P. infestans*. Such treatment causes the induction of systemic resistance to subsequent infection by *P. infestans* (Heller and Gessler, 1986). A similar induced resistance against *P. nicotianae* occurs in Samsun NN tobacco plants after TMV inoculation (McIntyre and Dodds, 1979). Until this study, none of the induced proteins was shown to inhibit *P. infestans* in vitro. As shown here, this may be explained by the hydrophobic nature of AP24 and the necessity to optimize the bioassay conditions for both the growth of *P. infestans* and protein solubility.

The fact that AP24 accumulates in the induced plants and inhibits the germination and growth of *P. infestans* suggests a possible role for AP24 in the induced resistance to this fungus and possibly other fungi. In tobacco, the majority of osmotin is located in the plant cell as insoluble protein bodies. There is also evidence for the presence of osmotin in the cytoplasm but to what extent is uncertain (Singh et al., 1987a). It is conceivable that in vivo the cytoplasmic osmotin (AP24) inhibits the growth of an invading hyphae or the development of a functional haustorium. Based on the in vitro assay, an AP24 concentration as low as 0.4 μM could be sufficient to inhibit the fungus. Because of the coenocytic nature of the hyphae, any lysis occurring in such an interaction could disrupt the growth of the entire thallus.

Further studies are needed to determine whether the growth of other fungi such as *P. nicotianae* is inhibited by AP24 and whether AP24 accumulates systemically in tobacco and tomato after localized induction. Microscopic studies using labeled antisera against AP24 may show whether the protein becomes associated with the invading hyphae during an incompatible interaction.

METHODS

Biological Materials

Phytophthora infestans, isolate 88069, was obtained from Dr. L. Davidse, Wageningen, The Netherlands. For maintenance and

sporangia formation, the fungus was grown at 20°C in the dark on rye agar medium (Caten and Jinks, 1968).

Tobacco (*Nicotiana tabacum* cv Samsun NN) and tomato (*Lycopersicon esculentum* cv Moneymaker) were grown at 24°C in an artificially illuminated room (12,000 Lux at plant height) with a 16-hr photoperiod. For protein induction in tobacco, 2-month-old plants were inoculated with TMV using carborundum (60 mesh). The plants were incubated for 7 days to allow necrotic lesion formation. The inoculated leaves were then harvested and stored at −80°C. Induction in tomato was achieved by inoculating leaves with zoospores of *P. infestans* according to the method described by Heller and Gessler (1986). Plants were incubated at 15°C and 100% relative humidity until lesions were formed (3 days to 4 days), then the plants were incubated for 4 days at 22°C and 75% relative humidity. The leaves were then harvested and stored at −80°C.

Sporangial Assay

To assay the effects of protein solutions on the growth of *P. infestans*, a method was designed using microtiter dishes. Microtiter dishes, 24-well or 96-well, were prepared by pipetting into the wells 250 μL or 50 μL of potato dextrose agar, respectively. Sporangia of *P. infestans* were suspended in water and added to the wells; 500 to 700 sporangia (in 50 μL) to the 24-well dishes and 100 to 200 sporangia (in 25 μL) to the 96-well dishes. Protein solutions tested in the assay were dialyzed against assay buffer (15 mM $KHPO_4$, pH 6.0, 20 mM NaCl) and filter sterilized (0.22 μm) before adding to the wells. As controls, protein solutions were boiled for 10 min. The microtiter dishes were incubated in the dark at 20°C for 4 days or 5 days. Mycelia was stained with lactophenol cotton blue. Microscopic observations were made at several time points during the incubation period using an inverted microscope.

Protein Purification

Proteins were extracted and purified by a modification of the method described by Kauffmann et al. (1987). TMV-infected tobacco leaves (400 g) or *P. infestans*-induced tomato leaves (400 g) were homogenized at 4°C in a Waring blender with 600 mL of 0.5 M NaOAc, pH 5.2, 0.1% β-mercaptoethanol, and active charcoal (1 g/100 g of leaves). The homogenate was filtered through cheesecloth, the filtrate centrifuged at 3000*g* for 15 min, and the supernatant centrifuged for 50 min at 20,000*g*. The resulting supernatant was passed through a Sephadex G-25 (medium coarse; Pharmacia) column (12 × 60 cm) equilibrated with 20 mM NaOAc, pH 5.2. The eluted protein solution was incubated overnight at 4°C before centrifuging for 50 min at 20,000*g*. The resulting supernatant was loaded onto an S-Sepharose (Fast Flow, Pharmacia) column (5 × 5 cm), and the adsorbed proteins were eluted with a linear 0 M to 0.4 M NaCl gradient. Fractions containing lysis activity against *P. infestans* were pooled, dialyzed against 1 M $(NH_4)_2SO_4$, 50 mM KPO_4, pH 7, and loaded onto a fast protein liquid chromatography-phenyl-superose column (HR5/5; Pharmacia). The adsorbed proteins were eluted using a decreasing $(NH_4)_2SO_4$ gradient. The final purification was obtained by pooling the fractions containing the lysis activity and rechromatographing on the phenyl-superose column under the same conditions.

Protein Analysis

Protein concentrations were determined by the method of Bradford (1976). Electrophoretic analysis was performed using 12.5% SDS-polyacrylamide gels (SDS-PAGE) according to the method of Laemmli (1970) and gels were stained with Coomassie Brilliant Blue G-250 (Neuhoff et al., 1988). For proteinase K treatment, protein samples in 30 mM Tris, pH 8, 1 mM $CaCl_2$, were incubated with 1.5 mg/mL proteinase K for 15 hr at 37°C. Immunoblotting methods were as described previously (Sijmons et al., 1990), and the antiserum used was produced in rabbit against PR-5 from tobacco (E.J.S. Meulenhoff, unpublished results). β-1,3-Glucanase and chitinase measurements were made using laminarin and ^{3}H-labeled chitin as substrates (Nelson, 1944; Molano et al., 1977). To obtain the N-terminal amino acid sequence, pure proteins were electrophoresed through 15% SDS-polyacrylamide gels and electroblotted to polyvinylidene difluoride membranes according to the procedure of Matsudaira (1987). The sequencing was done by Eurosequence, Groningen, The Netherlands, using Edman degradation on an Applied Biosystems 477A protein sequencer.

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REFERENCES

Bol, J.F., Linthorst, H.J.M., and Cornelissen, B.J.C. (1990). Plant pathogenesis-related proteins induced by virus infection. Annu. Rev. Phytopathol. **28,** 113–138.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72,** 248–254.

Bryngelsson, T., and Gréen, B. (1989). Characterization of a pathogenesis-related, thaumatin-like protein isolated from barley challenged with an incompatible race of mildew. Physiol. Mol. Plant Pathol. **35,** 45–52.

Carr, J.P., and Klessig, D.F. (1989). The pathogenesis-related proteins of plants. In Genetic Engineering: Principles and Methods, J.K. Setlow, ed (New York: Plenum Press), pp. 65–109.

Caten, C.E., and Jinks, J.C. (1968). Spontaneous variability of single isolates of *P. infestans*. I. Cultural variation. Can. J. Bot. **46,** 329–349.

Cohen, Y., and Kuć, J. (1981). Evaluation of systemic resistance to blue mold induced in tobacco leaves by prior stem inoculations with *Peronospora tabacina*. Phytopathology **71,** 783–789.

Cornelissen, B.J.C., Hooft van Huijsduijnen, R.A.M., and Bol, J.F. (1986). A tobacco mosaic virus-induced tobacco protein is homologous to the sweet-tasting protein thaumatin. Nature **321,** 531–532.

Dean, R.A., and Kuć, J. (1987). Immunisation against disease: The plant fights back. In Fungal Infection of Plants, G.F. Pegg and P.G. Ayres, eds (Cambridge University Press, Cambridge), pp. 383–410.

Doke, N., Ramirez, A.V., and Tomiyama, K. (1987). Systemic induction of resistance in potato plants against *Phytophthora infestans* by local treatment with hyphal wall components of the fungus. J. Phytopathol. **119,** 232–239.

Edens, L., Heslinga, L., Klok, R., Ledeboer, A.M., Maat, J., Toonen, M.Y., Visser, C., and Verrips, C.T. (1982). Cloning of cDNA encoding the sweet-tasting plant protein thaumatin and its expression in *Escherichia coli*. Gene **18,** 1–2.

Grosset, J., Marty, I., Chartier, Y., and Myer, Y. (1990a). mRNAs newly synthesized by tobacco mesophyll protoplast are wound inducible. Plant Mol. Biol. **15,** 485–496.

Grosset, J., Meyer, Y., Chartier, Y., Kauffmann, S., Legrand, M., and Fritig, B. (1990b). Tobacco mesophyll protoplast synthesize 1,3-β-glucanase, chitinases and "osmotins" during in vitro culture. Plant Physiol. **92,** 520–527.

Heller, W.E., and Gessler, C. (1986). Induced systemic resistance in tomato plants against *Phytophthora infestans*. J. Phytopathol. **116,** 323–328.

Jenns, A.E., and Kuć, J. (1980). Characteristics of anthracnose resistance induced by localized infection of cucumber with tobacco necrosis viruses. Physiol. Plant Pathol. **17,** 81–91.

Kauffmann, S., Legrand, M., Geoffroy, P., and Fritig, G. (1987). Biological function of "pathogenesis-related" proteins: Four PR proteins of tobacco have 1,3-β-glucanase activity. EMBO J. **6,** 3209–3212.

King, G.J., Hussey, C.E., Jr., and Turner, V.A. (1986). A protein induced by NaCl in suspension cultures of *Nicotiana tabacum* accumulates in whole plant roots. Plant Mol. Biol. **7,** 441–449.

King, G.J., Turner, V.A., Hussey, C.E., Jr., Wurtele, E.S., and Lee, S.M. (1988). Isolation and characterization of a tomato cDNA clone which codes for a salt-induced protein. Plant Mol. Biol. **10,** 401–412.

Laemmli, U.K. (1970). Cleavage of the structural proteins during the assembly of the head of the bacteriophage T4. Nature **227,** 680–685.

Legrand, M., Kauffmann, S., Geoffroy, P., and Fritig, B. (1987). Biological function of pathogenesis-related proteins: Four tobacco pathogenesis-related proteins are chitinases. Proc. Natl. Acad. Sci. USA **84,** 6750–6754.

Matsudaira, P. (1987). Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. J. Biol. Chem. **262,** 10035–10038.

Mauch, F., Mauch-Mani, B., and Boller, T. (1988). Antifungal hydrolases in pea tissue. II. Inhibition of fungal growth by combinations of chitinase and β-1,3-glucanase. Plant Physiol. **88,** 936–942.

McIntyre, J.L., and Dodds, J.A. (1979). Induction of localized and systemic protection against *Phytophthora parasitica* var. *nicotianae* by tobacco mosaic virus infection of tobacco hypersensitive to the virus. Physiol. Plant Pathol. **15,** 321–330.

Molano, J., Durán, A., and Cabib, E. (1977). A rapid and sensitive assay for chitinase using tritiated chitin. Anal. Biochem. **83,** 648–656.

Neale, A.D., Wahleithner, J.A., Lund, M., Bonnett, H.T., Kelly, A., Meeks-Wagner, D.R., Peacock, W.J., and Dennis, E.S. (1990). Chitinase, β-1,3-glucanase, osmotin, and extensin are expressed in tobacco explants during flower formation. Plant Cell **2,** 673–684.

Nelson, M. (1944). A photometric adaptation of the Somoqui method for the determination of glucose. J. Biol. Chem. **153,** 375–380.

Neuhoff, V., Arold, N., Taube, D., and Ehrhardt, W. (1988). Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie brilliant blue G-250 and R-250. Electrophoresis **9,** 255–262.

Ouchi, S. (1983). Induction of resistance or susceptibility. Annu. Rev. Phytopathol. **21,** 289–315.

Parent, J., and Asselin, A. (1987). Acidic and basic extracellular pathogenesis-related leaf proteins from fifteen potato cultivars. Phytopathology **77,** 1122–1125.

Pierpoint, W.S., Tatham, A.S., and Pappin, D.J.C. (1987). Identification of the virus-induced protein of tobacco leaves that resembles the sweet-protein thaumatin. Physiol. Mol. Plant Pathol. **31,** 291–298.

Pierpoint, W.S., Jackson, P.J., and Evans, R.M. (1990). The presence of a thaumatin-like protein, a chitinase and a glucanase among the pathogenesis-related proteins of potato (*Solanum tuberosum*). Physiol. Mol. Plant Pathol. **36,** 325–338.

Richardson, M., Valdes-Rodriguez, S., and Blanco-Labra, A. (1987). A possible function for thaumatin and a TMV-induced protein suggested by homology to a maize inhibitor. Nature **327,** 432–434.

Schlumbaum, A., Mauch, F., Vögeli, U., and Boller, T. (1986). Plant chitinases are potent inhibitors of fungal growth. Nature **324,** 365–367.

Sequeira, L. (1983). Mechanisms of induced resistance in plants. Annu. Rev. Microbiol. **37,** 51–79.

Sijmons, P.C., Dekker, B.M.M., Schrammeijer, B., Verwoerd, T.C., van den Elzen, P.J.M., and Hoekema, A. (1990). Production of correctly processed human serum albumin in transgenic plants. Bio/Technology **8,** 217–221.

Singh, N.K., Bracker, C.A., Hasegawa, P.M., Handa, A.K., Buckel, S., Hermodson, M.A., Pfankoch, E., Regnier, F.E., and Bressan, R.A. (1987a). Characterization of osmotin. A thaumatin-like protein associated with osmotic adaptation in plant cells. Plant Physiol. **85,** 529–536.

Singh, N.K., LaRosa, P.C., Handa, A.K., Hasegawa, P.M., and Bressan, R.A. (1987b). Hormonal regulation of protein synthesis associated with salt tolerance in plant cells. Proc. Natl. Acad. Sci. USA. **84,** 739–743.

Singh, N.K., Nelson, D.E., Kuhn, D., Hasegawa, P.M., and Bressan, R.A. (1989). Molecular cloning of osmotin and regulation of its expression by ABA and adaptation to low water potential. Plant Physiol. **90,** 1096–1101.

Van Loon, L.C. (1985). Pathogenesis-related proteins. Plant Mol. Biol. **4,** 111–116.